

New insights into a bacterial metabolic and detoxifying association responsible for the mineralization of the thiocarbamate herbicide molinate

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A novel pathway of molinate mineralization promoted by a defined mixed culture composed of five bacteria (named ON1 to ON5) was proposed previously. Evidence was obtained of a metabolic association between *Gulosibacter molinivorax* ON4^T, capable of molinate breakdown, and the remaining bacteria. In the present study, the role of each isolate in that metabolic association was further explored and the possible synergistic effect of all the bacterial isolates for the stability of the mixed culture is discussed. The cleavage of the molinate thioester bond, whether occurring under aerobic or anaerobic conditions, releases ethanethiol (S-ethyl moiety) and an azepane moiety derivative, identified as azepane-1-carboxylic acid. This azepane moiety is degraded, in the presence of oxygen, by *Pseudomonas* strains ON1 and ON3 and *G. molinivorax* ON4^T. Ethanethiol, which inhibits *G. molinivorax* ON4^T, is consumed by *Pseudomonas* strain ON1 and *Stenotrophomonas maltophilia* ON2. Although a two-member mixed culture of *G. molinivorax* ON4^T and *Pseudomonas* strain ON1 was able to promote the aerobic mineralization of molinate, after 20 successive transfers of the five-member mixed culture in mineral medium with molinate, none of these isolates were lost. The results obtained indicate that the whole mixed culture may have a higher fitness than the two-member culture, even when the basic degradative and cross-protection functions are assured.

INTRODUCTION

In nature, micro-organisms live in communities with different levels of complexity in terms of phylogenetic and metabolic diversity. In these communities, the stable coexistence of the micro-organisms results from different types of interaction, leading its members to function as a biological unit (Pelz *et al.*, 1999; Kato *et al.*, 2005). External perturbations, such as the introduction of large quantities of xenobiotics into the environment, create strong selective pressures. One expected reaction to such a stimulus is the establishment of communities able to degrade these compounds. The elucidation of the contribution of individual members of a microbial community to metabolic degradative

pathways may shed light on the biochemical transformations taking place and on the interrelationships responsible for the stable coexistence of the organisms. Several studies reporting co-operation among consortium members during the degradation of pollutants have shown different modes of microbial interaction. The literature available shows that the distribution of metabolic steps among consortium members may rely upon different forms of association, namely cross-feeding (e.g. De Souza *et al.*, 1998; Dejonghe *et al.*, 2003), detoxification (e.g. Feigl & Knackmuss, 1993; Pelz *et al.*, 1999) or supply of nutritional requirements (e.g. Hay *et al.*, 2001; Sorensen *et al.*, 2002).

The study of the evolution of degradative pathways has been another area of interest, encouraged by the need to deal with environmental contamination with xenobiotics. Some evidence suggests that an original metabolic association of various micro-organisms may evolve to a single-organism-based process (e.g. De Souza *et al.*, 1998). Such a transformation

Abbreviations: ACA, azepane-1-carboxylic acid; DGGE, denaturing gradient gel electrophoresis; DOC, dissolved organic carbon; HMI, hexamethyleneimine; HMQC, heteronuclear multiple quantum coherence; SPME-GC-FID, solid-phase micro-extraction-gas chromatography-flame ionization detection.

can be explained on the basis of horizontal gene transfer, leading to the assembly of different catabolic mobile genetic elements in a single cell. The origination of new catabolic routes by the rearrangement and combination of pre-existing genes of different micro-organisms has been reported (Tsuda *et al.*, 1999; Top *et al.*, 2002; Nojiri *et al.*, 2004). Thus, the description of metabolic pathways before the occurrence of horizontal transfer of catabolic genes may provide insights into the evolution of microbial degradation of xenobiotic compounds.

In a previous report (Barreiros *et al.*, 2003) we showed that a defined mixed culture composed of five bacterial isolates (mixed culture DC) could mineralize the herbicide molinate without the accumulation of degradation products. Isolates ON1 and ON3 were identified as *Pseudomonas chlororaphis* and *Pseudomonas nitroreducens*, respectively, isolate ON2 as *Stenotrophomonas maltophilia* and isolate ON5 as *Achromobacter xylosoxidans*; isolate ON4, not affiliated to any validly named taxon, represented a new genus and species, *Gulosibacter molinativorax*, within the family *Microbacteriaceae* (Manaia *et al.*, 2004). In mixed culture DC, *G. molinativorax* ON4^T was able to degrade molinate into ethanethiol and another compound that could not be detected but that was presumed to be an azepane derivative of the herbicide (Barreiros *et al.*, 2003). In spite of this degradative activity, *G. molinativorax* ON4^T was not able to grow in axenic culture with molinate at concentrations above 2 mM, an effect that was abolished when the Gram-negative members of the mixed culture were grown in a vial placed in the headspace of the growth vessel. This observation, along with the monitoring of sulphur compounds (ethanethiol and diethyl disulphide) formed in the headspace of the axenic but not in the mixed culture, suggested a detoxifying association between *G. molinativorax* ON4^T and its commensals. In this study, the fate of the azepane derivative and the role of each of the five members of culture DC in molinate mineralization were further investigated.

METHODS

Chemicals. Molinate (azepan-1-yl-ethylsulphanylmethanone, C₉H₁₇NOS) of 97% purity was obtained from Herbex, Produtos Químicos (Estoril, Portugal). Ethanethiol (C₂H₆S), diethyl disulphide (C₄H₁₀S₂), ethyl methyl sulphide (C₃H₈S), hexamethylenimine (HMI) (C₆H₁₃N), caprolactam (C₆H₁₁NO) and 6-aminohexanoic acid (C₆H₁₃NO₂) were obtained from Sigma-Aldrich. All other reagents were analytical grade from commercial sources.

Growth media and culture conditions. Culture DC and isolates ON1–ON5 were grown, in axenic or defined mixed cultures, in mineral medium B (Barreiros *et al.*, 2003) supplemented with molinate or other substrate, at 30 °C and 120 r.p.m. Inocula of culture DC were grown in medium B with 4 mM molinate and those of axenic cultures were grown on Luria–Bertani medium (LB) with agar (2% w/v), supplemented with 1 mM molinate (modified LA) to maintain the selective pressure in the medium.

The anaerobic degradation of molinate by *G. molinativorax* ON4^T and by culture DC was assayed in 100 ml PTFE-sealed flasks containing 75 ml medium B supplemented with 1 mM molinate and 0.01%

(w/v) potassium nitrate, and incubated under a nitrogen atmosphere for 15 days. Samples were collected over time and were analysed for biomass, molinate, dissolved organic carbon (DOC) and ethanethiol contents.

The ability of each member of culture DC to use the azepane moiety derivative azepane-1-carboxylic acid (ACA) resulting from molinate degradation was assessed by incubating each individual organism in the cell-free liquid phase of anaerobic resting cells of *G. molinativorax* ON4^T. ACA was produced by incubating resting cells of *G. molinativorax* ON4^T in sterile saline solution supplemented with 2 mM molinate, under a nitrogen atmosphere, at 30 °C and 120 r.p.m. for 12 h, as described below. The suspension was centrifuged (15 500 g, 10 min); the supernatant was filter-sterilized (0.2 µm pore size) and supplemented with medium B components and magnesium sulphate (0.4 mM) as a sulphur source. Growth was monitored over 7 days.

The ability of culture DC and its members to use different substrates as carbon and nitrogen sources was tested in medium B supplemented with 0.4 mM magnesium sulphate and 5.5 mM HMI, caprolactam or 6-aminohexanoic acid. When necessary, the medium was supplemented with 4 mM ammonium sulphate (as nitrogen source) or with 0.2 g yeast extract l⁻¹ (as source of growth factors). Cultures were incubated aerobically in 100 ml screw-capped Erlenmeyer flasks with Teflon-lined caps. Cell growth was monitored over 7 days.

To compare the molinate mineralization efficiency of culture DC and of the mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T, these cultures were grown aerobically in 100 ml PTFE-sealed flasks containing 20 ml medium B with 4 mM molinate. Culture samples were collected at regular intervals and analysed for biomass, molinate and DOC contents. The presence of molinate S-ethyl and azepane moieties was also analysed, in the headspace (SPME-GC-FID) and in the liquid phase of cultures (NMR), respectively. An axenic culture of *G. molinativorax* ON4^T and uninoculated medium were analysed similarly, as controls.

To assess the stability of mixed culture DC, cells were grown in medium B with 4 mM molinate for 5 days at 30 °C, stored at 4 °C for 8–20 days, transferred to fresh medium and incubated for a further 5 days at 30 °C. Culture composition was assessed by PCR-denaturing gradient gel electrophoresis (DGGE) after a total of 20 successive culture transfers.

Resting cell assays. The isolates were grown on modified LA medium at 30 °C, for 1 or 3 days, centrifuged, washed and resuspended in phosphate buffer 54 mM, pH 7.2 (PB), supplemented with the substrate to test. For NMR analysis, PB was replaced by 50 mM NaCl. Cell densities corresponding to 4 g cell dry weight l⁻¹ were used in all assays. To evaluate the ability of each isolate to consume ethanethiol, resting cell assays were performed in PB supplemented with 0.4 mM of this sulphur compound. Resting cells of culture DC were used as positive control and uninoculated buffer with ethanethiol was used to assess abiotic losses. Sulphur compound content was measured in the headspace (SPME-GC-FID). To obtain and/or identify the azepane derivative, resting cells of *G. molinativorax* ON4^T were incubated in saline solution supplemented with 2 mM molinate under a nitrogen atmosphere, at 30 °C and 120 r.p.m. Samples were collected over time and the liquid phase was analysed for molinate content (HPLC) or for detection and identification of molinate metabolites (NMR).

To evaluate if the use of HMI and caprolactam by *G. molinativorax* ON4^T were induced processes, resting cells pregrown with 2 mM of the azepane moiety derivative of molinate (ACA) were incubated in PB with 2 mM HMI and/or caprolactam. Abiotic losses were assessed in uninoculated buffer with the same concentration of HMI and/or caprolactam. Samples were collected over time and analysed by NMR or HPLC to follow HMI or caprolactam degradation, respectively. *Pseudomonas* strains ON1 and ON3 were also tested for ACA-HMI induction of caprolactam degradation, using the same procedure.

Analytical procedures. Cell growth was monitored spectrophotometrically (OD₆₁₀; Philips PU-8620UV/VIS spectrophotometer) and cell dry weight was obtained via a calibration curve (Barreiros *et al.*, 2003). Molinate and DOC contents in culture supernatants were analysed (HPLC and total organic carbon) as described before (Barreiros *et al.*, 2003). Ethanethiol was quantified in the liquid phase using the colorimetric method described by Dias & Weimer (1998). The analysis of ethanethiol, diethyl disulphide and ethyl methyl sulphide in the headspace of cultures or resting cells assays was performed by SPME-GC-FID as described by Barreiros *et al.* (2003).

NMR analysis. Proton spectra were recorded on Bruker DRX500 or AMX300 spectrometers operating, respectively, at 500.13 MHz and 300.13 MHz for ¹H, equipped with 5 mm diameter inverse detection broadband probe heads (with and without XYZ gradients). In both cases water pre-saturation was applied. Acquisition parameters on DRX500: spectral width, 10 kHz; pulse width, 7 ms (60° flip angle); data points, 64K; repetition delay, 1 s; number of transients, 100. Acquisition parameters on AMX300: spectral width, 5 kHz; pulse width, 4 ms (70° flip angle); data points, 16K; repetition delay, 1.5 s; number of transients, 100. Carbon spectra were recorded on a Bruker DRX500 operating at 125.77 MHz for ¹³C and equipped with a ¹H/¹³C dual-probe head (5 mm diameter). Acquisition parameters: spectral width, 31.5 kHz; pulse width, 6 ms (70° flip angle); data points, 64K; repetition delay, 10 s; number of transients, 5500. Proton broadband decoupling was applied during acquisition time only (1.04 s). All the spectra were acquired at a constant temperature of 27 °C. Chemical shifts were referenced to tetramethylsilane at 0.0 p.p.m. (¹H) and methanol at 49.3 p.p.m. (¹³C), used as external references.

Denaturing gradient gel electrophoresis. Total genomic DNA was extracted based on the method described by Cashion *et al.* (1977) from 0.35 mg biomass of culture DC or of each isolate. A 200 bp fragment (based on the reference strain *Escherichia coli* bases 338 and 518) of the 16S rRNA gene was amplified using the bacterial 16S rDNA primers forward 338F_{GC}, containing a GC clamp (5'-CGC CCG CCG GCG GCG GGC GGG GCG GGG GCA CGG GGG GACTCC TAC GGG AGG CAG CAG-3'), and reverse 518R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.*, 1993). Reaction mixtures (50 µl) were prepared as described by Henriques *et al.* (2006). The PCR conditions were: 5 min at 94 °C, 30 cycles of (30 s at 92 °C, 30 s at 55 °C, 30 s at 72 °C), and 7 min at 72 °C. PCR products were separated in a 8 % (w/v) polyacrylamide gel with a denaturing gradient ranging from 30 to 55 % (where 100 % denaturant contained 7 M urea and 40 % formamide), running initially at 20 V for 20 min, and then at 200 V for 5.5 h at 60 °C. DGGE bands corresponding to each culture DC isolate were excised from the gel, eluted with 20 µl ultrapure water, reamplified by PCR with the same primers, cleaned (GFX PCR DNA and Gel Band Purification kit, Amersham Biosciences) and subsequently sequenced with primer 518R. The sequences were determined using a model ABI 3700 DNA analyser (Applied Biosystems), and their quality was checked manually using the BioEdit software (Hall, 1999). These sequences were compared with those obtained for the 16S rRNA gene of each isolate (accession numbers: *P. chlororaphis* ON1, AJ306832; *S. maltophilia* ON2, AJ306833; *P. nitroreducens* ON3, AJ306834; *G. molinativorax* ON4^T, AJ306835; *A. xylosoxidans* ON5, AJ306836).

Kinetic models. Specific cell growth and molinate degradation rates were calculated during the exponential growth phase according to the first-order kinetic model described by Barreiros *et al.* (2003). The same data were fitted to the Haldane equation:

$$\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_i}}$$

where μ and μ_{\max} are, respectively, the specific and the maximum

specific growth rates, S is the molinate concentration, and K_s and K_i are the saturation and inhibition constants, respectively.

RESULTS AND DISCUSSION

Identification of the azepane moiety derivative of molinate

In order to gain new insights into the complete mineralization of molinate by mixed culture DC, the azepane moiety derivative originated by the cleavage of molinate by *G. molinativorax* ON4^T was identified. In previous studies this ring moiety could not be detected, a fact that was explained on the basis of its possible rapid use, as a carbon and/or nitrogen source, by *G. molinativorax* ON4^T and, eventually, other members of the mixed culture (Barreiros *et al.*, 2003). However, the limitation of the growth conditions and analytical methods (HPLC and GC-MS) used could also hamper the detection of the azepane moiety. In fact, it was observed that under anaerobic conditions mixed culture DC degraded 90 % (w/w) of the added molinate, whereas only 28 % of the dissolved organic carbon (DOC) was consumed after 80 h of incubation (Correia *et al.*, 2006). These data suggested the accumulation of the molinate azepane moiety, although it could not be detected by HPLC or GC-MS. Therefore, it was hypothesized that, in the absence of oxygen, the hydrolysis of the molinate thioester bond could occur as long as *G. molinativorax* ON4^T was viable, although the further degradation of the metabolites was not possible. This hypothesis was tested by culturing mixed culture DC and *G. molinativorax* ON4^T anaerobically. Under this condition, mixed culture DC was not able to grow, although the herbicide, at an initial concentration of ~160 mg l⁻¹, was slowly degraded to concentrations below the detection limit (0.9 mg l⁻¹). Ethanethiol, a major product of molinate breakdown by *G. molinativorax* ON4^T, was not detected in the medium. In spite of this, after 15 days of incubation the initial DOC content was reduced by only 30 % (Table 1). In a parallel experiment, *G. molinativorax* ON4^T showed different results: only 45 % (w/w) of the molinate was degraded; ethanethiol accumulated in the medium (18 mg l⁻¹ in the liquid phase), and the initial DOC content was reduced by only 12 % (Table 1). These final DOC values were consistent with those that would result from partial molinate hydrolysis. These results suggest that under anaerobic conditions *G. molinativorax* ON4^T maintains its ability to break down the molinate thioester bond. Apparently, none of the mixed culture DC members is able to further metabolize the azepane derivative that accumulates in the medium. In contrast, ethanethiol was consumed by the Gram-negative isolates of mixed culture DC. Thus, the toxic effect of that thiol on *G. molinativorax* ON4^T was attenuated and it was able to proceed with molinate hydrolysis.

The findings obtained with anaerobic cultures gave a good hint to proceed with the characterization of the azepane moiety. Thus, the anaerobic degradation of molinate by resting cells of *G. molinativorax* ON4^T was followed by

Table 1. Anaerobic degradation of molinate by *G. molinivorax* ON4^T and mixed culture DC grown in mineral medium B with molinate

HPLC detection limit of 0.9 mg l⁻¹ for molinate; colorimetric method detection limit of 0.9 mg l⁻¹ for ethanethiol; ND, Not determined.

Culture	0 days			7 days			15 days			
	Cell dry weight (mg l ⁻¹)	Molinate (mg l ⁻¹)	DOC (mg l ⁻¹)	Cell dry weight (mg l ⁻¹)	Molinate (mg l ⁻¹)	DOC (mg l ⁻¹)	Cell dry weight (mg l ⁻¹)	Molinate (mg l ⁻¹)	DOC (mg l ⁻¹)	Ethanethiol (mg l ⁻¹)
<i>G. molinivorax</i> ON4 ^T	35	167	121	26	73	110	7	75	106	18
Culture DC	39	156	117	33	12	101	25	<0.9	82	<0.9
Uninoculated medium with 1 mM molinate	–	162	121	–	134	112	–	151	108	<0.9
Uninoculated medium with 1 mM ethanethiol	–	–	ND	–	–	ND	–	–	ND	18

¹H-NMR (Fig. 1). During incubation, the resonances assigned to molinate ($-S-CH_2CH_3$ at 1.20 p.p.m.; $-S-CH_2CH_3$ at 2.82 p.p.m.; azepane ring at 3.47, 1.70, 1.63 and 1.48 p.p.m.) decreased and the formation of a product attributed to the molinate azepane moiety was observed. Small amounts of free ethanethiol remained in solution and were detected ($HS-CH_2CH_3$ at 1.12 p.p.m.; $HS-CH_2CH_3$ at 3.60 p.p.m.). After

6 h incubation, all the molinate was converted into degradation products that were not further modified, even after 30 h incubation. This procedure proved to be a good process to obtain high amounts of the azepane-containing compound and, hence, to proceed with its characterization by ¹H and ¹³C NMR (Fig. 2a, b). ¹H and ¹³C-NMR chemical shifts characteristic of the new compound, identified as azepane-1-carboxylic acid (ACA), observed in saline solution (50 mM NaCl) at physiological pH are indicated in Table 2. In Fig. 2(a), resonances in the proton spectrum are shifted to the low-field region due to ionic strength of the solution (approx. 5 M NaCl) upon lyophilization of the liquid phase of anaerobic resting cells of *G. molinivorax* ON4^T with molinate after 12 h incubation. This is believed to be the first report of ACA as an intermediate of molinate degradation,

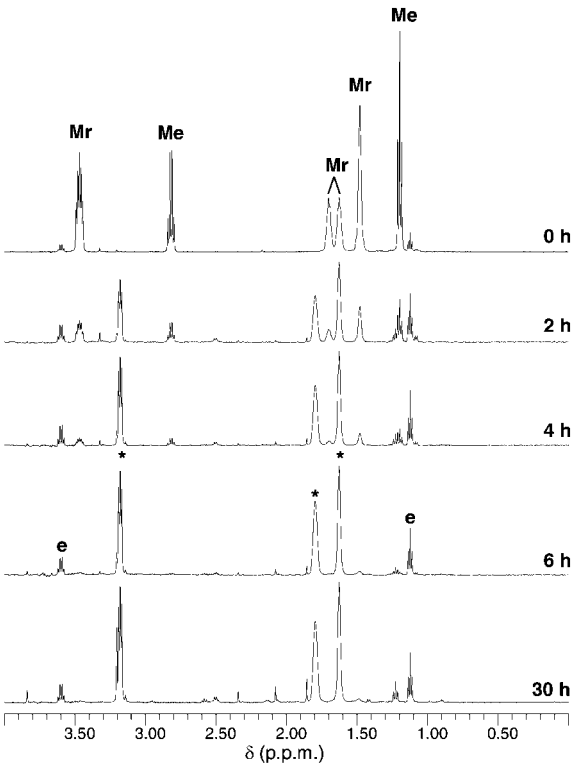


Fig. 1. ¹H-NMR spectra of the liquid phase of resting cells of *G. molinivorax* ON4^T in 50 mM NaCl solution with molinate (2 mM), under anaerobic conditions. Assignment of the observed resonances: Mr, molinate azepane ring; Me, molinate S-ethyl group; e, ethanethiol; *, new anaerobic end product.

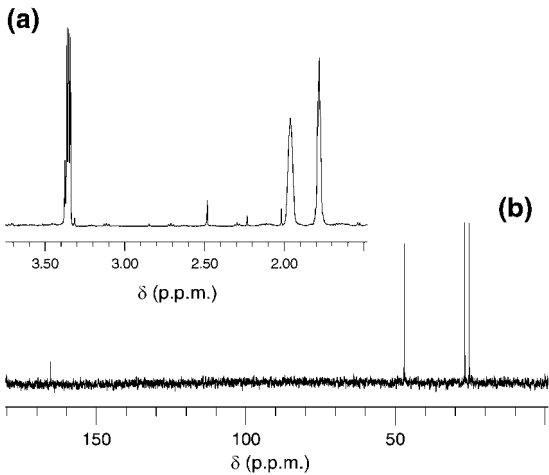
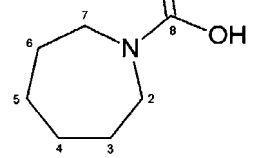


Fig. 2. ¹H-NMR (a) and ¹³C-NMR (b) spectra of azepane-1-carboxylic acid, obtained as described in Fig. 1 (12 h), lyophilized and resuspended in D₂O prior to acquisition of spectra. The HMQC 2D-NMR technique was used to confirm C–H correlations (data not shown).

Table 2. ^1H and ^{13}C -NMR chemical shifts of ACA in saline solution (50 mM NaCl)

	Position	δ ^1H (p.p.m.)	δ ^{13}C (p.p.m.)
	2, 7	3.18	47.04
	3, 6	1.80	26.78
	4, 5	1.63	25.30
	8	—	165.32

suggesting that *G. molinativorax* ON4^T may use a unique process to degrade this herbicide.

Degradation of ACA by members of the mixed culture

ACA was degraded by resting cells of *Pseudomonas* strains ON1 and ON3 and of *G. molinativorax* ON4^T after 25 h incubation, as this compound was not detected in the liquid phase (results not shown). ACA also supported, as carbon and nitrogen source, and in the absence of any additional organic growth factor, the aerobic growth of these organisms, with specific growth rates of 0.27 h^{-1} (ON1), 0.22 h^{-1} (ON3) and 0.13 h^{-1} (ON4). In contrast, *S. maltophilia* ON2 and *A. xylosoxidans* ON5 were not able to grow at the expense of ACA, either as carbon and nitrogen source, or as carbon source only. Apparently, this inability was not due to auxotrophy, as growth was not observed even in the presence of $0.2\text{ g yeast extract l}^{-1}$.

McClung *et al.* (1994) described a microbiological process of degradation of 1-ethylsulphonyl-*N,N*-dipropylformamide (EPTC), which consisted of the hydrolysis of the thioester bond, with release of ethanethiol, dipropylamine and CO_2 . Hypothesizing an equivalent transformation for molinate, the organisms able to use ACA – *Pseudomonas* strains ON1 and ON3 and *G. molinativorax* ON4^T – would promote its decarboxylation into hexamethyleneimine (HMI), which could be further consumed as a source of carbon and nitrogen. Such a supposition was consistent with previous reports on molinate biodegradation that refer to the release of the azepane moiety in the form of HMI as a degradation product of the intermediate molinate sulfoxide (Thomas & Holt, 1980; Imai & Kuwatsuka, 1986). In order to confirm the hypothesis that further molinate degradation could occur via HMI, this compound, and other commercially available possible analogues of the degradation metabolites of ACA, were assayed as growth substrates. In fact, all the isolates able to degrade ACA were also able to grow at the expense of HMI. However, in the *Pseudomonas* strains this process was constitutive, whereas in *G. molinativorax* ON4^T it was subject to ACA induction. These data suggest that ACA decarboxylation to HMI, by analogy with the metabolic pathway proposed by McClung *et al.* (1994), represents the next step of molinate mineralization. According to Wackett & Hershberger (2001), HMI degradation may follow one of two alternative pathways, via caprolactam or via an open-ring

aldehyde metabolite. *Pseudomonas* strains ON1 and ON3 were unable to grow at the expense of caprolactam, even after induction with HMI. Thus, HMI degradation in these strains may proceed via 2-hydroxy-HMI, followed by isomerization into an open-ring aldehyde metabolite and further oxidation to 6-aminohexanoic acid. In contrast, caprolactam was degraded by *G. molinativorax* ON4^T, although only after successive induction with ACA and HMI. This finding hints that HMI degradation in *G. molinativorax* ON4^T occurs via a distinct pathway, where caprolactam is formed and further converted into 6-aminohexanoic acid. This last compound, common to both HMI degradation routes, supported the growth of both pseudomonads and of *G. molinativorax* ON4^T.

Degradation of the S-ethyl moiety of molinate

Ethanethiol and diethyl disulphide, formed during the initial breakdown of molinate, are toxic to *G. molinativorax* ON4^T. However, in mixed culture DC this toxic effect is avoided because *P. chlororaphis* ON1 and *S. maltophilia* ON2 are able to consume these sulphur compounds (Barreiros *et al.*, 2003). Further investigation of the degradative performance of these two isolates revealed that *P. chlororaphis* ON1 consumed ethanethiol and diethyl disulphide at a higher rate than *S. maltophilia* ON2 (Table 3). These results were confirmed by the specific degradation rates of resting cells of these isolates with 1 mM ethanethiol. *P. chlororaphis* ON1 degraded this compound at a rate approximately 10 times higher [$0.36 \pm 0.13\text{ mg ethanethiol (g cell dry weight)}^{-1}\text{ h}^{-1}$] than *S. maltophilia* ON2 [$0.03 \pm 0.009\text{ mg ethanethiol (g cell dry weight)}^{-1}\text{ h}^{-1}$]. *P. nitroreducens* ON3 was also able to transform ethanethiol, although it was observed to be a conversion only, as no depletion of the added carbon occurred. In fact, approximately 75 % of the added ethanethiol was converted to ethyl methyl sulphide. Given that none of the culture DC members was able to convert or to degrade the ethyl methyl sulphide, and considering the high rates of ethanethiol transformation during molinate mineralization (Barreiros *et al.*, 2003), this product is probably not formed by *P. nitroreducens* ON3, when *S. maltophilia* ON2, and particularly *P. chlororaphis* ON1, are also present. Taken together, these results suggest that, in mixed culture DC, the ethanethiol formed during molinate cleavage can be consumed by *P. chlororaphis* ON1 and *S. maltophilia* ON2, although the first, as a more rapid degrader, may compete for this substrate.

Evaluation of the stability of mixed culture DC

Fig. 3 shows a putative degradation pathway for molinate and the flow of carbon, nitrogen and sulphur through the different members of culture DC, as suggested by the results obtained in the present study. According to this proposed pathway, molinate can be mineralized by *G. molinativorax* ON4^T and *P. chlororaphis* ON1. First, molinate is hydrolysed into ethanethiol and ACA by *G. molinativorax* ON4^T, then ethanethiol is consumed by *P. chlororaphis* ON1, whereas ACA supports the growth of both strains. The behaviour of the mixture of these

Table 3. Degradation of sulphur compounds by resting cells of mixed culture DC isolates with 0.4 mM ethanethiol

Values are the mean \pm SD of three independent experiments. SPME-GC-FID detection limit of 0.02 mM for ethanethiol, 0.0005 mM for diethyl disulphide and 0.02 mM for ethyl methyl sulphide.

Assay	Time (h)	Ethanethiol		Diethyl disulphide		Ethyl methyl sulphide		% Carbon total
		mM	% Carbon*	mM	% Carbon*	mM	% Carbon*	
<i>P. chlororaphis</i> ON1	0	0.386 \pm 0.031	95.6 \pm 2.6	0.009 \pm 0.005	4.4 \pm 2.6	<0.02	—	100.0
	24	<0.02	—	<0.0005	—	<0.02	—	—
	48	<0.02	—	<0.0005	—	<0.02	—	—
<i>S. maltophilia</i> ON2	0	0.384 \pm 0.026	95.5 \pm 2.8	0.009 \pm 0.006	4.5 \pm 2.8	<0.02	—	100.0
	24	0.216 \pm 0.047	53.7 \pm 11.0	0.063 \pm 0.028	31.7 \pm 14.4	<0.02	—	85.4 \pm 5.2
	96	0.188 \pm 0.064	46.6 \pm 15.0	0.044 \pm 0.019	21.7 \pm 9.0	<0.02	—	68.3 \pm 8.5
<i>P. nitroreducens</i> ON3	0	0.379 \pm 0.013	94.5 \pm 3.6	0.011 \pm 0.007	5.5 \pm 3.6	<0.02	—	100.0
	24	0.246 \pm 0.032	61.3 \pm 8.2	0.035 \pm 0.020	17.3 \pm 10.1	0.067 \pm 0.010	25.2 \pm 3.9	103.8 \pm 3.5
	96	0.144 \pm 0.061	35.8 \pm 15.4	0.007 \pm 0.006	3.2 \pm 2.9	0.196 \pm 0.036	73.1 \pm 13.4	112.2 \pm 4.2
<i>G. molinativorax</i> ON4 ^T	0	0.394 \pm 0.017	96.5 \pm 1.4	0.007 \pm 0.002	3.5 \pm 1.4	<0.02	—	100.0
	24	0.388 \pm 0.011	95.2 \pm 5.3	0.005 \pm 0.001	2.3 \pm 0.4	<0.02	—	97.5 \pm 5.6
	96	0.357 \pm 0.007	87.7 \pm 3.9	0.017 \pm 0.002	8.4 \pm 1.0	<0.02	—	96.1 \pm 4.3
<i>A. xylosoxidans</i> ON5	0	0.386 \pm 0.023	95.4 \pm 0.8	0.009 \pm 0.002	4.6 \pm 0.8	<0.02	—	100.0
	24	0.297 \pm 0.022	73.4 \pm 4.1	0.040 \pm 0.006	20.1 \pm 4.3	<0.02	—	93.5 \pm 5.6
	96	0.252 \pm 0.010	62.3 \pm 3.7	0.060 \pm 0.011	29.3 \pm 4.2	<0.02	—	91.6 \pm 1.5
Culture DC	0	0.379 \pm 0.016	93.7 \pm 3.4	0.013 \pm 0.007	6.3 \pm 3.4	<0.02	—	100.0
	24	<0.02	—	<0.0005	—	<0.02	—	—
	48	<0.02	—	<0.0005	—	<0.02	—	—
Abiotic control	0	0.389 \pm 0.022	93.7 \pm 2.9	0.013 \pm 0.005	6.3 \pm 2.9	<0.02	—	100.0
	24	0.317 \pm 0.067	76.1 \pm 14.5	0.050 \pm 0.031	24.5 \pm 15.8	<0.02	—	100.6 \pm 1.7
	96	0.213 \pm 0.030	51.3 \pm 6.7	0.099 \pm 0.019	47.8 \pm 10.1	<0.02	—	99.1 \pm 6.6
Abiotic control with autoclaved cells	0	0.366 \pm 0.001	91.6 \pm 6.1	0.017 \pm 0.014	8.4 \pm 6.1	<0.02	—	100.0
	24	0.199 \pm 0.043	50.4 \pm 13.6	0.087 \pm 0.026	43.1 \pm 9.8	<0.02	—	93.4 \pm 4.9
	96	0.133 \pm 0.009	33.4 \pm 4.1	0.119 \pm 0.014	59.6 \pm 3.4	<0.02	—	93.0 \pm 1.7

*Percentage of carbon in the form of ethanethiol (C₂H₆S), diethyl disulphide (C₄H₁₀S₂) or ethyl methyl sulphide (C₃H₈S), respectively.

isolates in the presence of 4 mM molinate was compared with that of mixed culture DC. The analysis of Fig. 4(a, b) shows that both mixed cultures were able to grow on molinate with concomitant DOC depletion to values of 20–30 mg l⁻¹. SPME-GC-FID analysis of the culture headspace and ¹H-NMR of the respective supernatant after 72 h incubation revealed that neither the S-ethyl moiety (ethanethiol and diethyl disulphide) nor ACA accumulated in the medium (results not shown). However, the comparison of the specific growth and molinate degradation rates of the two mixed cultures revealed that, although molinate was mineralized by the mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T, a possible substrate inhibition may occur. While the experimental data of growth and molinate degradation by culture DC fit a first-order kinetics [0.06 h⁻¹ and 208 mg molinate (g cell dry weight)⁻¹ h⁻¹, respectively], as described before (Barreiros *et al.*, 2003), the data corresponding to the mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T could be described by the Haldane equation (see Methods), a conventional model of substrate inhibition. The Haldane model parameters obtained for culture DC and the mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T (with K_s =

1 mg l⁻¹) were, respectively, μ_{\max} 0.38 h⁻¹ and 1.39 h⁻¹, and K_i 102.0 mg l⁻¹ and 11.5 mg l⁻¹. The specific growth rate of the mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T increases from 0.026 h⁻¹ for initial molinate concentration of about 600 mg l⁻¹ up to 0.04 h⁻¹ after consumption of approximately half the initial molinate concentration.

Molinate mineralization by culture DC seems to be comparatively more efficient, suggesting a synergistic interaction among its members. This may explain the stability of culture DC, with maintenance of its five members even after successive subculturing in mineral medium with molinate. In fact, DGGE analysis of mixed culture DC after 20 successive transfers, over a period of more than 9 months, revealed that none of the five isolates was lost (Fig. 5), as confirmed by the comparison of the sequences of each excised band with those of the pure isolates of mixed culture DC.

An important aspect concerning the stability of mixed culture DC was the presence of two organisms with no apparent function in molinate degradation. One of these isolates was *S. maltophilia* ON2, able to use ethanethiol, although at low rates, and presumably with poor growth yields. The other

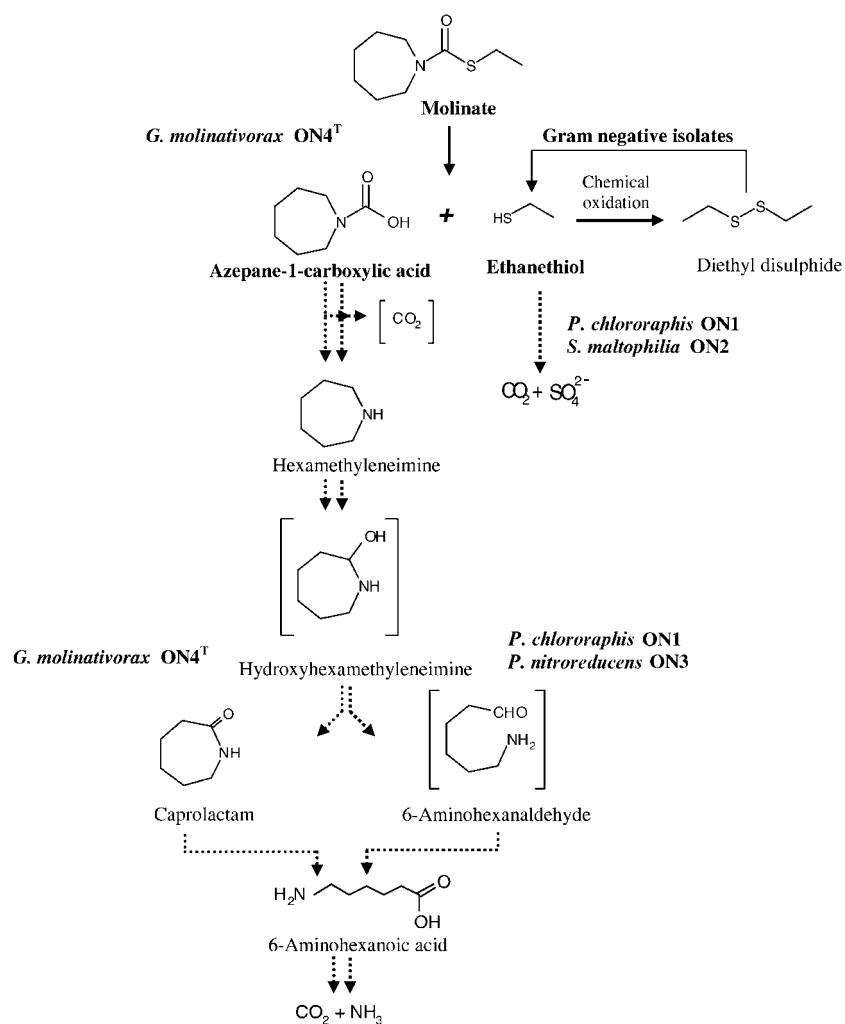


Fig. 3. Putative degradation pathway of molinate and assignment of metabolic functions of mixed culture DC members (reactions on the left, with dotted arrows, represent transformations by *G. molinativorax* ON4^T; reactions on the right, with bold dotted arrows, represent transformations by Gram-negative isolates). Compounds in brackets are presumed intermediates/products. Solid arrows represent experimentally documented reactions.

was *A. xylosoxidans* ON5, unable to grow in either the S-ethyl or azepane moieties of molinate. However, neither of these two isolates was lost after the successive transfers of mixed culture DC, a fact that suggests that the maintenance of these isolates in the mixed culture may be due to the use of extracellular metabolites or debris released by other mixed-culture members. Alternatively, a product of partial degradation of ACA could feed these isolates. However, this hypothesis seems unlikely, because ACA metabolism is presumably intracellular, as indicated by the fact that no other metabolite was detected in the ¹H-NMR spectra of resting cell assays of *Pseudomonas* strains ON1 and ON3 and *G. molinativorax* ON4^T. The presence of subsidiary members in stable microbial communities is probably commonplace in nature and has been referred to in other biodegradation studies. For example, Pelz *et al.* (1999) reported the stable co-existence of a member with no apparent function in a community in a chemostat fed with 4-chlorosalicylate. The stability of consortia for long periods may be based upon a complex set of microbial interrelationships, which may include synergistic interactions involving carbon sharing, consumption of toxic metabolites, commensalism and/or

competition (e.g. Pelz *et al.*, 1999; Christensen *et al.*, 2002; Dejonghe *et al.*, 2003; Kato *et al.*, 2005).

The observation that micro-organisms have evolved high catabolic potential for numerous pollutants has led to their intensive isolation and characterization, with the ultimate objective of establishing cost-effective bioremediation processes. The accumulation of knowledge on the metabolites/catalysts/genes involved in the breakdown of different organic functional groups permitted the conclusion that different micro-organisms would degrade different compounds of the same class of chemical structure by similar catabolic routes. This allowed for creation of databases of degradative pathways (e.g. University of Minnesota Biocatalysis/Biodegradation Database, <http://umbdd.ahc.umn.edu/>) of great utility for pre-viewing the catabolic routes of new isolates and/or pollutants. However, possibly due to the evolutionary potential and genetic flexibility of micro-organisms (Timmis & Pieper, 1999), there are still descriptions of novel pathways for the degradation of pollutants for which other catabolic routes were previously known (e.g. Pieper *et al.*, 2004). Apparently this is the case for molinate. Initially it was described as co-metabolizable by three

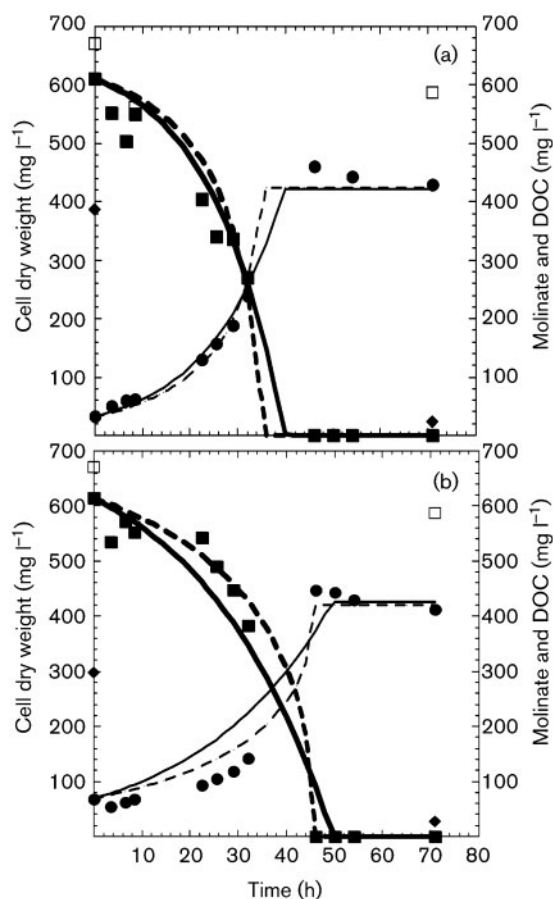


Fig. 4. Growth, molinate degradation and DOC reduction by mixed culture DC (a) and a mixture of *P. chlororaphis* ON1 and *G. molinativorax* ON4^T (b) in medium B with 4 mM molinate. ●, Cell dry weight; ■, molinate; ◆, DOC; □, molinate in uninoculated medium. Fittings of a first-order kinetic model for cell dry weight and molinate are shown by full lines and bold full lines, respectively; fittings of the Haldane model for cell dry weight and molinate are shown by dashed lines and bold dashed lines, respectively.

possible degradative routes: (1) the oxidation of the sulphur atom with production of molinate sulfoxide and molinate sulphone; (2) the oxidation of the azepane moiety with production of hydroxy- and oxomolinate; or (3) the oxidation of the S-ethyl moiety with production of molinate alcohol and molinate acid (Soderquist *et al.*, 1977; Thomas & Holt, 1980; Golovleva *et al.*, 1981; Imai & Kuwatsuka, 1986). Among those compounds, only trace amounts of 2-oxomolinate were detected in culture DC grown with molinate as the only source of carbon, nitrogen and energy (Correia *et al.*, 2006), supporting the hypothesis that the herbicide is degraded by this mixed culture through a different catabolic route. We (Barreiros *et al.*, 2003; the present work) have described a new mineralizing pathway, involving the hydrolysis of the herbicide thioester bond by *G. molinativorax* ON4^T with the release of ethanethiol and ACA, which is apparently further degraded, via

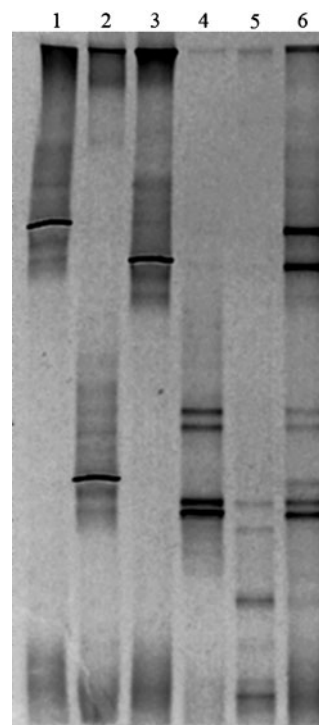


Fig. 5. DGGE profiles of mixed culture DC after 20 transfers in medium B with 4 mM molinate, and of each individual member as controls. Lanes: 1, *P. chlororaphis* ON1; 2, *S. maltophilia* ON2; 3, *P. nitroreducens* ON3; 4, *G. molinativorax* ON4^T; 5, *A. xylosoxidans* ON5; 6, 20th culture of mixture DC.

previously described mechanisms, by *Pseudomonas* strains ON1 and ON3 and *G. molinativorax* ON4^T.

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